

CIRCADIAN RHYTHMS: MECHANISM OF LUCIFERASE ACTIVITY CHANGES IN *GONYAULAX*

LAURA McMURRY AND J. W. HASTINGS

*Biological Laboratories, Harvard University, 16 Divinity Avenue,
Cambridge, Massachusetts 02138*

Gonyaulax polyedra is a bioluminescent dinoflagellate which exhibits a daily rhythmic fluctuation in its capacity for bioluminescence (Hastings and Sweeney, 1958). When grown using a LD 12:12 cycle (12 hours light alternating with 12 hours darkness), the period of the bioluminescence rhythm is exactly 24 hours; the maximum luminescence is in the middle of the dark period. However, rhythmicity will persist in constant laboratory conditions, where it assumes its "circadian" period, close to but not exactly 24 hours. Under these conditions the phase of the rhythm is independent of that of the earth's daily cycle (Aschoff, 1956; Bünnig, 1967).

Little is known about the mechanism of circadian rhythms or about the biochemistry involved in their expression.

In *Gonyaulax* activity of the extractable soluble enzyme luciferase displays a circadian rhythm which correlates well with the bioluminescence rhythm of the intact cell. Luciferase, which catalyzes *in vitro* luminescence via oxidation of a low molecular weight substrate "*Gonyaulax* luciferin," has been partly purified and characterized (Bode and Hastings, 1963; Fogel and Hastings, 1971). The luciferase activity in the supernatant resulting from centrifuging a cell homogenate for about 30 minutes at $27,000 \times g$ fluctuates rhythmically with time of extraction, both for cells from a light-dark cycle (Hastings and Bode, 1962) and for cells from constant dim light (Fig. 1). Hastings and Bode (1962) reported that this rhythm was not one of total protein extractability and that the day-night difference in luciferase activity per mg protein was retained after an 8-fold purification.

A number of other cases in which enzymatic activity varies from night to day are known (Sanwal and Krishnan, 1960; Potter, Gebert, Pitot, Peraino, Lamar, Leshner, and Morris, 1966; Rapoport, 1966; Civen, Ulrich, Trimmer, and Brown, 1967; Hardeland, 1969; Sweeney, 1969), but in only a few cases (Hardeland, Sweeney) has the molecular basis been examined, and in no case has it been well defined.

We report here further experiments to discover the immediate biochemical basis for the cycling in *Gonyaulax* luciferase activity. We also discuss the contribution of the luciferase rhythm to the bioluminescence rhythm of the intact cell.

MATERIALS AND METHODS

Gonyaulax polyedra is a photosynthetic, bioluminescent, armored marine dinoflagellate about 40μ in diameter. Two strains were used. The non-axenic strain was that used by DeSa (1964) and reported by him to have been isolated in

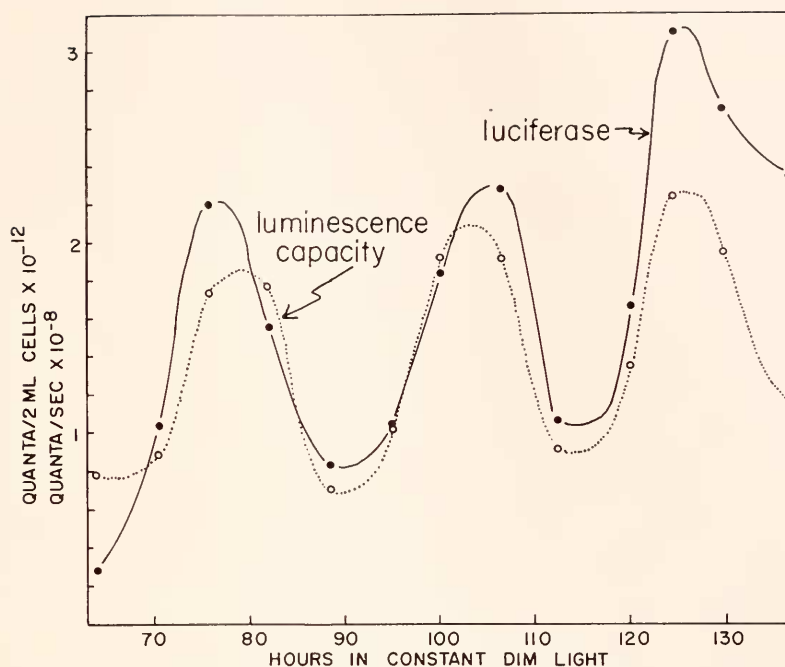


FIGURE 1. Luciferase activity rhythm and luminescence capacity rhythm in constant light (160 footcandles), 22° C. The culture was transferred into constant light at the end of a light period. Cells were pelleted from 80 ml of culture by a 1.5 minute centrifugation at speed 3 in an International clinical centrifuge (more convenient for harvesting small volumes of culture than filtration through a Büchner funnel), suspended in 16 ml 0.05 M tris, 0.01 M EDTA, pH 8, 0.005 M DTT, and homogenized once. The cell debris was removed by 5 minutes' centrifugation at $2000 \times g$ and reextracted with 8 ml. The first supernatant and second homogenate were combined and centrifuged 15 min at $27,000 \times g$. The supernatant was assayed for luciferase in assay mixture #1.

1952 by Dr. B. M. Sweeney. Cultures were maintained as previously described (Fogel and Hastings, 1971). The second strain (used only for the experiment shown in Figure 1) was an axenic clone derived in the laboratory of Dr. Robert Guillard, Woods Hole Oceanographic Institute, from a strain isolated by Dr. Sweeney in 1960. The data in Figure 1 accompanied other measurements, not pertinent here, which required an axenic culture. The second strain was maintained in "f/2" medium ("f/2" medium is the "f" medium of Guillard and Ryther (1962) diluted in half with sea water). Both strains have similar luciferase rhythms.

Luminescence was measured by a photomultiplier and amplifier as described by DeSa (1964). The instrument was calibrated at 490 nm using the secondary luminescence standards of Hastings and Weber (1963). The intensity of fluorescent lights used in growing cells was measured with a Weston illumination meter model 756, quartz filter.

Under LD 12:12 growth conditions, we found the luciferase activity of "mid-night" extracts (made after 5 to 7 hours of darkness) to be about 10 times that

of "mid-day" extracts (made after 5 to 7 hours of light), while under constant light the rhythm had a smaller amplitude (Fig. 1). Therefore we used LD conditions and mid-day and mid-night extraction times to assure both a large difference between minimum and maximum luciferase activities and predictability for the phase of the rhythm. A culture of 3000 to 10,000 cells/ml (uniform in any given experiment) was harvested by filtration on a Büchner funnel (except in Fig. 1) and the cells were suspended in either cold pH 6.0 extraction buffer (0.05 M sodium potassium phosphate) or cold pH 8.0 extraction buffer (0.05 M tris, 0.01 M EDTA) with 0.001 to 0.005 M DTT. Extraction in the first buffer yields luciferase of approximately 35,000 molecular weight, the second 150,000 M.W. (Fogel and Hastings, 1971). Ten ml or more of extraction buffer was used per 800 ml of culture. The cell suspension was passed twice through a stainless steel hand emulsifier (Fisher Scientific Co., catalog #11-504-2000). The homogenate was centrifuged in the HB4 swinging bucket rotor in a Sorvall refrigerated centrifuge as described in the figure legends and the pellet discarded.

In a study of luciferase extractability, guanidine was used in the extraction medium. Cells were extracted by homogenization in 5 M guanidine hydrochloride, 0.005 M DTT, pH 6.7 at 22° C. As the control, cells were extracted with the pH 6 extraction buffer at 4° C. "Day" cells were harvested 5.5 hours after lights on and "night" cells 4.5 hours after lights off. The crude homogenate was centrifuged at $25,000 \times g$ for 10 minutes to give a supernatant. The luciferase, denatured by guanidine, was renatured by 1/25 dilution into 0.05 M tris, 0.01 M EDTA, 0.001 M DTT, 0.1 mg/ml BSA, pH 8.0 at 4° C, where complete recovery took about 5 hours. Control samples were similarly diluted. Assays were done in assay mixture #2.

To obtain luciferin for the luciferase assay, cultures from the day, or from the night after an hour's exposure to bright light (Bode, DeSa and Hastings, 1963), were harvested as described above and cells suspended in buffer in a boiling water bath. The buffer was 0.0025 M tris, 0.0005 M EDTA, pH 8.0; about 1.5 ml per flask of culture (800 ml) was used. After 2 minutes, the solution was chilled, made 0.005 M in DTT, centrifuged 30 minutes at $27,000 \times g$, and the supernatant frozen and stored in 1 ml portions at -57° C.

Two different reaction mixtures were used for the luciferase assay, using a volume of 2 ml in both cases. Assay mixture #1 (Bode and Hastings, 1963) was 1 M ammonium sulfate, 0.1 M tris-maleate, 0.4 mg/ml BSA, 0.0025 M EDTA, pH 6.4 to 6.7. Assay mixture #2 (Fogel and Hastings, 1971) was simpler and so preferred in later experiments; it was 0.2 M sodium phosphate, 0.1 mg/ml BSA, pH 6.2. The assay was carried out by adding (in either order) luciferin and luciferase in prompt sequence; the reaction was initiated by the last addition. The intensity was recorded at a fixed time after initiation (about 10 sec) and was proportional to the luciferase concentration over the range assayed. Light emission without added luciferin was negligible. Unless otherwise specified, the assays were done in duplicate or triplicate with an average error of 11%.

Luminescence capacity refers to the amount of light emitted by the intact cell when stimulated by mechanical or chemical means. In these experiments, two ml of cell culture was placed in a 20 ml vial above a photomultiplier and 1 ml 0.06 N acetic acid was injected into the vial. The burst of light thus elicited was inte-

grated electronically for 5 sec, at which time emission was complete. Assays were done in triplicate and had an average error of 4%.

The activity of luciferases from "day" and "night" cells were compared after centrifugation in sucrose density gradients. Pig heart lactate dehydrogenase purchased from Sigma Chemical Corporation was used as a marker enzyme to control for variations in sedimentation velocity from one gradient to the next. One hundred μ l of luciferase from a pH 8 tris extraction was mixed with 10 μ l lactate dehydrogenase (0.21 mg/ml) and layered on top of a 4.5 ml, 5–16% convex exponential sucrose gradient, made with 0.05 M tris, 0.01 M EDTA, 0.005 M DTT, pH 8.0. Gradients were spun 23.9 hours at 37,400 rpm in a SW 39 rotor in a Beckman ultracentrifuge model L2. Gradient tubes were impaled on a syringe needle and fractions of 10 drops were collected in small iced tubes containing 25 μ l 0.05 M tris, 0.01 M EDTA, 5% sucrose, 0.1 mg/ml BSA. Two luciferase assays were done on each fraction; 50 μ l of the fraction was used for each. Assay mixture #2 was used. To assay for lactate dehydrogenase, sodium pyruvate to make 0.00076 M and NADH to make 0.058 mg/ml were freshly added to 0.03 M Na_2HPO_4 , pH 7.4; then to 1 ml of this in a cuvette was added a 25 μ l gradient sample. The contents of the cuvette were mixed and the change in optical density at 340 nm per minute was measured on the 0.1 slideware of a Cary 15 recording spectrophotometer.

Abbreviations used are: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; LD 12:12, a light/dark cycle with 12 hours of light alternating with 12 hours of darkness; NADH, reduced nicotinamide adenine dinucleotide; and tris, tris-(hydroxymethyl)-aminomethane.

RESULTS

A trivial explanation for the luciferase activity rhythm, namely, that total protein might manifest such a rhythm in extractability, had been stated to be untrue (Hastings and Bode, 1962). Any individual enzyme such as luciferase, however, might still be bound more securely than proteins generally during the day than during the night and so be retained better by the cell debris. We made a preliminary check for such selective retention by assaying the crude cell homogenate (which includes the cell debris) for luciferase in the standard luciferase assay. Extractions made in pH 6.0 phosphate buffer showed that there was 2 to 3 times more luciferase activity in a crude cell homogenate than in the supernatant after the cell debris had been removed by centrifugation. This difference, however, was found in extracts made during the night as well as during the day. These measurements therefore gave no evidence that selective retention of assayable luciferase by cell debris during the day explained the luciferase rhythm.

The possibility remained that only a fraction (say 10%) of the luciferase in "day" cells was similar in extractability and activity to that in "night" cells while the bulk of luciferase in day cells was rendered both inextractable and inactive by its location within the cell. Were this so, the luciferase might become assayable if it could be released from its location. Therefore we tried different mechanical methods of extraction as well as different extraction media in an effort to bring the activity of day extracts up to the level of that of night extracts.

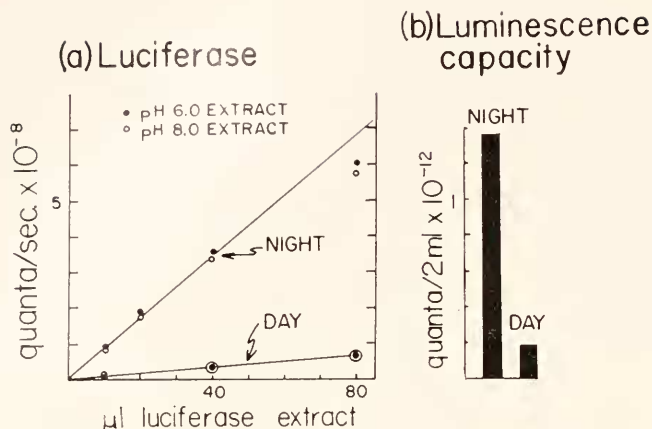


FIGURE 2. (a) Comparison of luciferase activity in extracts made in 0.05 M phosphate buffer, pH 6 ("pH 6 extract") and in 0.05 M tris, 0.01 M EDTA, pH 8 ("pH 8 extract") during both the day and the night. Fifteen hours before the first harvest, cultures of cells were combined and divided between two flasks. Extractions were made with 0.001 M DTT 7 hours after lights off (NIGHT) and 3.5 hours after lights on (DAY), using one flask for each time, one half of the flask for each of the two extraction pH's. The extract was spun 3 min at $2,000 \times g$ to remove cell debris, then 15 min at $20,000 \times g$. Assay mixture #1 was used, final pH 6.7, one assay per point. (b) Luminescence capacity measured in the same experiment just prior to cell harvest.

In all cases we failed to accomplish this. Typical day-night differences in luciferase activity were observed whether the extraction was done by stirring the cells in buffer or by emulsification with the Fisher emulsifier. A Ten-Broeck glass homogenizer was found to be only about half as effective as the emulsifier in releasing luciferase during *both* the day and the night. In addition, we found similar activities in extracts made in phosphate buffer at pH 6 and in tris buffer with EDTA at pH 8 at any given time, while the day activity was still about 10% of the night activity (Fig. 2). These findings are also of interest because it had been shown that the extraction medium determines which of two molecular weight forms of luciferase would be obtained, 35,000 (phosphate buffer, pH 6) or 150,000 (tris buffer with EDTA, pH 8) (Fogel and Hastings, 1971).

Another variation in the extraction medium involved the use of guanidine, which disrupts noncovalent bonds (which might be responsible for holding day luciferase more firmly in the cell) (Tanford, Kawahara, Lapanje, Hooker, Zarlengo, Salahuddin, Aune, and Takagi, 1967). Extraction in the emulsifier with 5 M guanidine did not release proportionately more luciferase activity from "day" cells than it did from "night" cells in comparison to pH 6 phosphate extraction buffer. (See Methods for details. The luciferase extracted in guanidine is denatured but is restored to complete activity by subsequent dilution in tris buffer at pH 8.) Extraction in guanidine apparently detached all the assayable luciferase from the cell debris, since the same recoverable activity was found in the guanidine supernatant above the cell debris as was found in the guanidine (and phosphate) crude homogenates before the cell debris was removed by centrifugation. Extraction in

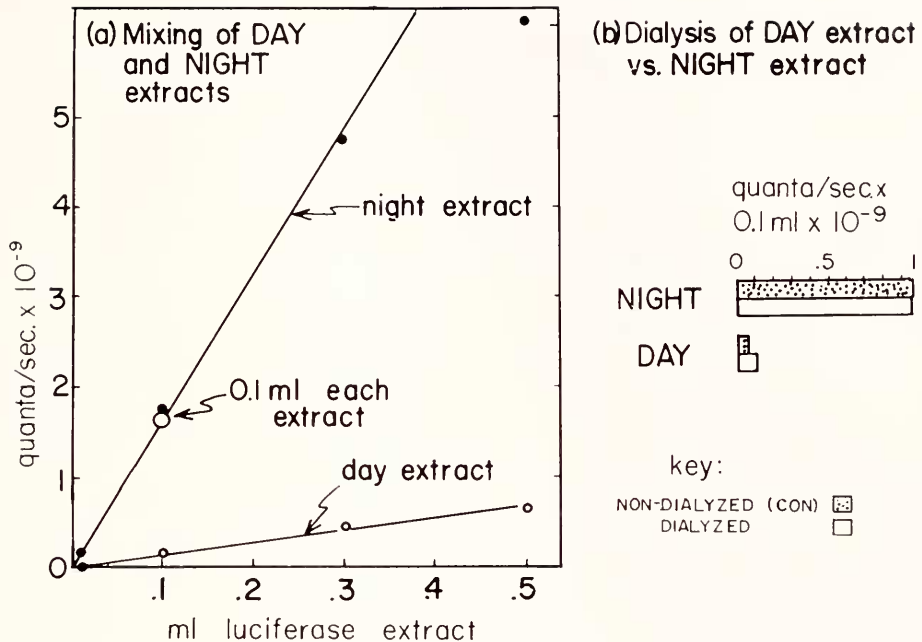


FIGURE 3. Two experiments testing for presence of an inhibitor or activator of luciferase in extracts made during the day and during the night. Five days before harvest time a flask from constant dim light was diluted with new medium; half these cells were put on one LD 12:12 cycle, the other half on the same cycle, 12 hours out of phase. Cells were harvested 6 hours after lights on or lights off and extracted in 0.05 M tris, 0.01 M EDTA, pH 8.0 (0.005 M DTT). The homogenate was centrifuged 1 hour at $23,000 \times g$. (a) Mixing aliquots of day and night extracts to check whether activities were additive. $(0.5-x)$ ml extraction buffer and x ml luciferase extract were added to assay mixture #1 containing 50 μ l luciferin. (b) Dialysis of day extract against night extract. 1.5 ml day extract was put inside a dialysis bag and dialyzed 13 hours at 4° against 3 ml night extract; activity changes were compared to those of undialyzed extracts containing pieces of dialysis bag (controls); 0.1 ml luciferase was assayed.

phosphate buffer, on the other hand (as mentioned above), apparently detached only $\frac{1}{3}$ to $\frac{1}{2}$ of the available luciferase activity.

From these experiments there is no evidence that the luciferase rhythm is due to a rhythm in luciferase extractability. We now turn our attention to the possible presence of activators or inhibitors in the extracts.

Were an activator present in the night extract in a greater than stoichiometric amount, this extract would enhance the day extract's activity; conversely, a day extract, if it contained an inhibitor, should reduce the activity of the night extract. The total activity of such mixtures of day and night extracts was, however, approximately the sum of that of the two constituents (Fig. 3a). Several other experiments confirmed this; the slight inhibition seen in Figure 3a fell within experimental error.

If such an activator or inhibitor were present but in only stoichiometric amounts, the activity of mixtures *would* be additive. If it were, however, not tightly bound,

TABLE I

Effect of ammonium sulfate precipitation upon the activity of luciferase from the day and from the night. (The same luciferase extract was used as for Figure 3. The supernatant from the 1 hour's centrifugation was made 70% in ammonium sulfate over a 5 minute period and stirred then for 10 minutes at 4° C. The luciferase was pelleted at 15,000 rpm in a Sorvall SS34 in 30 minutes and resuspended in 1 ml extraction buffer. Ten μ l of extract was assayed for luciferase in the manner of Figure 3, A.S. = ammonium sulfate precipitation)

Time at which extract was made	Total activity of extract: (quanta/sec)		% Recovery
	Before A.S.	After A.S.	
Day	12×10^9	6.7×10^9	56%
Night	120×10^9	72×10^9	60%

it should be removable upon purification of the enzyme. However, the difference between day and night luciferase activity (for the 150,000 molecular weight form) persisted after dialysis (even when day luciferase was dialyzed against night luciferase (Fig. 3b)), ammonium sulfate precipitation (Table I) and sucrose velocity gradient centrifugation (Fig. 4), each done on crude enzyme. Therefore, either no activator or inhibitor was present, or, if one was, it did not separate from its luciferase during these purification treatments. Separation might have failed to occur if the hypothetical inhibitor or activator were bound to the luciferase or if it behaved similarly to luciferase during purification.

DISCUSSION

Summarizing these experiments, the rhythm of luciferase activity in crude extracts of *Gonyaulax* cells from a LD 12:12 cycle does not appear to result from a rhythm of luciferase extractability. However we cannot completely exclude this possibility (for example, during the day, luciferase might be covalently bound to the cell debris and rendered inactive). Further, it does not appear to be caused by a rhythm in concentration of an activator or inhibitor molecule of any variety present in greater than stoichiometric amount; nor does it appear to be caused by a rhythm in dissociable activator or inhibitor molecule present in stoichiometric amounts unless such a molecule is similar to luciferase in its behavior upon dialysis, ammonium sulfate precipitation, and sucrose velocity gradient centrifugation.

The results thus rule out several explanations for the rhythmic change in luciferase activity. Two possible explanations appear to remain. One is that there are simply more luciferase molecules present in night cells than in day cells. Such a situation would imply large scale *de novo* synthesis and degradation processes *in vivo* not associated with growth. A method similar to that of Filner and Varner (1967) involving heavy isotopes C^{13} and N^{15} and sucrose velocity gradients was used to investigate this possibility (McMurry, 1971) but the results were not conclusive; they suggested that there may not be sufficient luciferase synthesis to support a hypothesis of complete *de novo* synthesis from amino acids.

A second explanation would involve a chemical moiety which attaches and

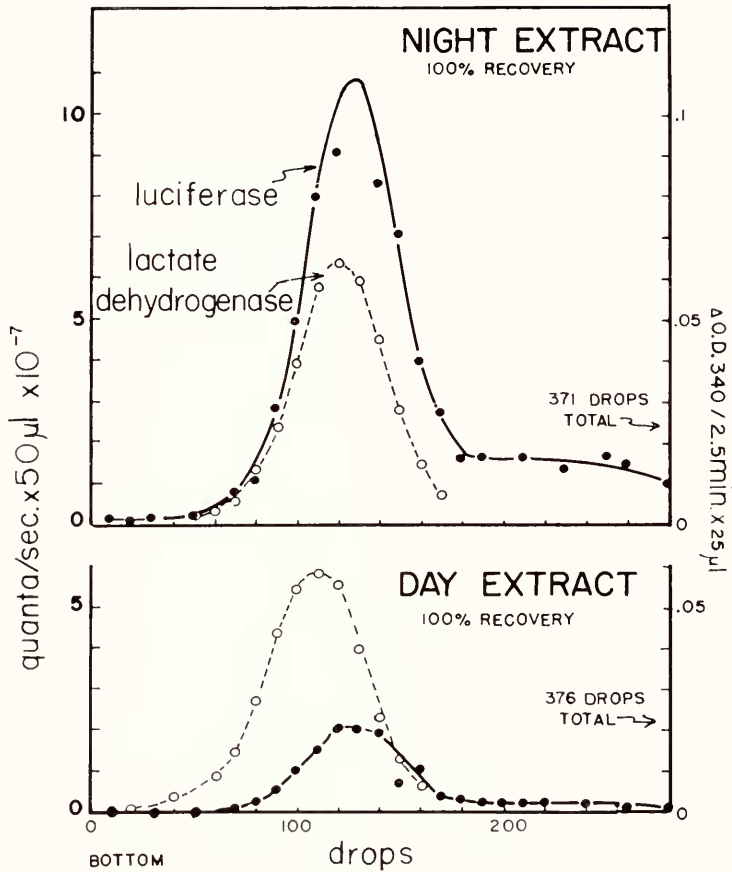


FIGURE 4. Behavior of luciferase activity from a day and a night extract during sedimentation of the extracts through sucrose gradients, showing persistence of the difference between day and night activities. Half of each of two 800 ml cultures was harvested 6 hours after lights on (DAY) and 4.5 hours after lights off (NIGHT), extracted in 0.05 M tris, 0.01 M EDTA, pH 8.0, 0.005 M DTT, centrifuged 40 min at $25,000 \times g$, and stored at -57°C . Gradients were run as described under Methods.

detaches, possibly covalently, to and from the "backbone" of the luciferase molecule during each circadian cycle. The presence of this moiety would alter the luciferase activity. Luciferase would thus occur in two forms, one more active than the other. In the absence of growth, the total number of luciferase "backbone" molecules would remain constant. One of two opposite situations might obtain: (1) the moiety might cause a 10-fold change in activity; every "day" luciferase molecule might then be 10% as active as every "night" molecule, in which case the active species would differ chemically from day to night; (2) the moiety might completely activate or inactivate; all of the molecules would be active at night while only 10% of the molecules would be active by day. In this case the active species would be the same from day to night.

For either situation, the moiety could be of any size or nature compared to the luciferase backbone, with appropriate effects upon the separation of backbone from backbone-plus-moiety during purification (the moiety could even be a cellular organelle). The moiety itself could undergo a daily *de novo* synthesis and destruction or it could be always present in the cell. The moiety could be a protein. It should be noted in this respect that an activity rhythm is seen for both the 150,000 and 35,000 molecular weight forms of luciferase (Fig. 2).

If there is any difference in the molecular weight between active day and night luciferase, it is not large (Fig. 4); the somewhat smaller sedimentation velocity for day luciferase seems to be at least partly a function of its lower activity, for if night enzyme is diluted to comparable activity, it has a similarly smaller sedimentation velocity (McMurry, 1971).

The rhythm of luminescence capacity parallels that of soluble luciferase activity (for example, see Fig. 1 and 2). What is the significance of this fact?

Luminescence capacity can be measured in two ways, by stimulating the cells with acid to emit a burst of light (as in this report), or by bubbling them with air to cause many individual bright flashes (Hastings and Sweeney, 1958). Values obtained with acid are two to three times higher than those obtained by bubbling, but the rhythms are otherwise apparently equivalent (McMurry, 1971); we may therefore in this discussion talk about cell flashes.

Over 95% of the light emitted during bubbling comes from cell flashes (McMurry, 1971). However, luminescent particles, the scintillons (DeSa, Hastings, and Vatter, 1963), rather than soluble luciferase, are believed to be responsible for cell flashes (Hastings, Vergin, and DeSa, 1966; Eckert and Reynolds, 1967; McMurry, 1971; Fogel and Hastings, 1972). Hence the question is likely one of the relationship between the soluble luciferase and the scintillons.

Scintillons can utilize free luciferin and likely also contain luciferase (Fogel, 1970; Fogel and Hastings, 1972). The luciferase extracted in soluble form may be in equilibrium *in vivo* with luciferase on the scintillons, or it may be solubilized from scintillons during extraction. In either case, if the amount of light which scintillons emit *in vivo* were for some reason proportional to their luciferase activity content, the soluble luciferase activity would reflect the luminescence capacity.

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SUMMARY

The bioluminescent marine dinoflagellate *Gonyaulax polyedra* manifests similar circadian rhythms of bioluminescence capacity and extractable luciferase activity, both with maxima during the night phase. The immediate biochemical basis of the luciferase rhythm was investigated, with the following findings:

(1) The rhythm was present no matter which of several mechanical extraction methods and extraction media (including 5 M guanidine) were employed. The rhythm was present even in a crude cell homogenate. Thus the rhythm is likely not one of extractability unless luciferase is inactivated while being covalently bound to cell debris during day phase.

(2) Mixing experiments, ammonium sulfate precipitation, dialysis, and sucrose velocity gradient centrifugation showed that no dissociable activator or inhibitor of luciferase caused the rhythm.

Two possible hypotheses remain untested: (a) the occurrence of *de novo* luciferase synthesis and destruction, (b) the attachment (perhaps covalent) and detachment of an activity-modifying moiety.

The luminescence capacity rhythm is primarily a rhythm of quantity of light from cell flashes. Cell flashes probably originate from extractable particles termed scintillons which flash during assay. The relationship of the luciferase rhythm to the luminescence capacity rhythm is discussed from this view.

Note added in proof: For recent findings on the luminescence capacity rhythm see R. Christianson and B. M. Sweeney, 1972. Sensitivity to stimulation, a component of the circadian rhythm in luminescence in *Gonyaulax*. *Plant Physiol.*, **49**: 994-997.

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